Comments of the International Fragrance Association North America (IFRA North America) on the Draft Hazard Identification Document (HID) "Evidence on the Carcinogenicity of Coumarin"

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Submitted to:

Office of Environmental Health Hazard Assessment (OEHHA)

Members of the Carcinogen Identification Committee (CIC)

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Comment of IFRA North America

I. Summary

The draft Hazard Identification Document (HID) identifies studies in mice, rats and hamsters that have evaluated the potential carcinogenicity of coumarin. As the HID notes, the quality and reliability of these studies varies. Many of these studies have significant limitations and shortcomings that limit their usefulness for purposes of Proposition 65. In order for the Carcinogen Identification Committee (CIC) to recommend listing coumarin, it must be "clearly shown through scientifically valid testing according to generally accepted principles to cause" cancer. These comments regarding the draft HID can be summarized with the following points:

Animal Evidence of Carcinogenicity: The results of the animal studies do not support a conclusion that coumarin is clearly shown to cause cancer. The results of the studies by NTP (1993) and Carlton et al. (1996) are briefly summarized in the table below.

Summary of NTP (1993) and Carlton et al. (1996) 2-year carcinogenicity studies of coumarin: clear evidence of carcinogenicity?

Study (species)	Sex	Results	Clear Evidence?
NTP (1993) B6C3F1 mice	M	↑ Lung adenomas (but not carcinomas) at high dose only	No
	F	↑ Lung adenomas and carcinomas at high dose only	Yes
Carlton (1996) CD-1 mice	M	No ↑ in tumors when adjusted for mortality; all within historical control incidence	No
	F	↑ hepatocellular tumors at low dose only	No
NTP (1993) F344/N rats	M	↑ Renal tubule adenomas (but not carcinomas) at low and middle dose only	No
	F	No ↑ in tumors at any dose	No
Carlton (1996) S-D rats	M	No ↑ in tumors except at high dose that greatly exceeded maximum tolerated dose (MTD)	No
	F	No ↑ in tumors except at high dose that greatly exceeded maximum tolerated dose (MTD)	No

↑ = statistically significant increase by pairwise comparison

NTP (1993): The only statistically significant increase in any malignant tumor in any carcinogenicity study of coumarin is the increase in in alveolar/bronchiolar carcinomas (as well as adenomas) in female B6C3F1 mice given the high dose of coumarin in the NTP gavage bioassay. In fact, NTP concluded that the increased incidence of lung tumors in high dose female mice was the only "clear evidence of carcinogenic activity" in its cancer bioassay in mice and rats. In male mice, NTP observed a statistically significant increase in alveolar/bronchiolar adenomas at the high dose, but coumarin had no effect on the incidence of alveolar/bronchiolar carcinomas (1/50, 1/50, 2/50, and 1/51 at 0, 50, 100, and 200 mg/kg, respectively). Importantly, B6C3F1 mice are particularly sensitive to lung tumors, which is attributed to the high metabolic rate of coumarin epoxidation in the Clara cell, and mouse lung tumors associated with coumarin are of questionable relevance to humans (Lake *et al.,* 1999; Born *et al.,* 1998; Vassallo *et al.,* 2004).

In male F344/N rats, NTP reported a statistically significant increase in renal tubule adenomas at the low and middle doses (but not at the high dose) that did not demonstrate a dose-response relationship. But, there was no evidence of an increase in renal tubule carcinomas among the male rats exposed to coumarin (0/49, 1/50, 0/51, and 0/50 at 0, 50, 100 and 200 mg/kg, respectively). In female rats, there was no statistically significant (by pairwise comparison) effect on renal tubule adenomas incidence of renal tubule adenomas (0/49, 0/50, 1/50, and 2/49 at 0, 50, 100, and 200 mg/kg, respectively). In addition, NTP reported that the trend test was not statistically significant for these benign tumors. However, OEHHA reported a trend test p value of 0.0489 for the incidence of renal tubule adenomas when OEHHA used the "number of tumor-bearing animals per number of animals alive at time of first occurrence of tumor (day 699)" in the denominator. OEHHA also reported that renal tubule adenomas are rare in female rats based on NTP's historical control data; however, it should be noted that the results in the coumarin bioassay were based on the results of single and step-sectioning (combined) of the kidneys whereas most historical controls data came from studies which did not employ step-sectioning. No renal tubule carcinomas were observed at any dose among the female rats in the NTP bioassay.

Carlton et al. (1996): A subsequent 2-year study in mice (CD-1) given coumarin in the diet did not confirm the findings in mice in the NTP bioassay. In contrast to the results of the NTP bioassay, no dose-related increase in lung adenomas or carcinomas was observed in female mice, despite the fact that Carlton included a higher dose level of coumarin than did NTP. In female mice, the draft HID reported a statistically significant increase in combined hepatocellular adenomas and carcinomas at the low dose, but not at the middle and high dose levels in the Carlton et al. study. The study authors stated that "all tumor incidences were within laboratory historic control values for this age and strain of mice."

In male mice, no significant increase in liver tumors was observed at any dose level; the incidence of hepatocellular adenomas and carcinomas combined was 20/52, 22/52, 19/52, and 12/52 at 0, 26, 86, and 280 mg/kg/day. The incidence of pulmonary carcinoma (but not adenoma) was statistically significantly increased among male mice administered the high dose of coumarin based on a Fisher pairwise comparison with controls conducted by OEHHA. However, the IARC Working Group stated that it "was aware of an unpublished company report in which statistical analyses had been applied to mortality-adjusted tumour rates. The Fisher's exact test for differences between treatment groups and Mantel's test for dose-related trends showed no treatment-related effect for any tumour type." Without the individual animal data survival and tumors, OEHHA would not have been able to evaluate the mortality-adjusted tumor rates.

In summary, in the Carlton mouse study, the incidence of tumors, including lung and liver tumors, did not exceed the historical control range at any dose in either sex. No dose-related effect on liver tumors was observed in mice of either sex. Although a statistically significant increase in pulmonary carcinoma was described in the draft HID among high dose males based on a statistical analysis conducted by OEHHA, there was no statistically significant effect when the tumor data were adjusted for mortality, as described by IARC. Thus, there was no clear evidence of a treatment-related effect on

lung, liver, or any other type of tumors in male or female CD-1 mice administered coumarin in the diet.

In rats, no significant increase in the incidence of any type of tumor was observed among male and female Sprague-Dawley rats exposed by Carlton et al. (1996) to dose levels of coumarin that did not exceed the Maximum Tolerated Dose (MTD). In comparison, a statistically significant increase in liver tumors was reported at the high dose, which clearly exceeded the MTD. Importantly, dose levels that exceed the MTD are not considered to represent "scientifically valid testing according to generally accepted principles." There is no question that the high dose exceeded the MTD. As stated in the HID, "mean body weight in the high-dose group was approximately 43% lower than controls at 52 weeks and 35% lower at 104 weeks." (OEHHA, 2017, p. 15). During the first 13 weeks of the study, the high dose male and female rats gained 266 and 102 grams less weight, respectively, than the control males and females. These animals were clearly compromised at the high dose. By the end of the study, male and female high dose rats gained 252 and 229 grams less, respectively, than the control males and females. Thus, the highest dose levels given to rats by Carlton et al. (1996) should not be considered "scientifically valid testing according to generally accepted principles."

Other Animal Studies: As detailed in the draft HID and in this submission, the Hagan et al. (1967), Bar and Griepentrog (1967), Griepentrog (1973), and Ueno and Hirono (1981) studies suffer from significant limitations. These studies are not adequate, and they should not be regarded as "scientifically valid testing according to generally accepted principles."

Epidemiological Evidence of Carcinogenicity: No epidemiological studies on the potential carcinogenicity of human exposure to coumarin were identified in recent literature searches conducted by OEHHA and IFRA North America. Thus, the human studies do not support a finding that coumarin is clearly shown to cause cancer.
It is noted that coumarin is naturally occurring in a number of plants, including many that are a common part of the human diet (e.g., strawberries, apricots, cherries). As

summarized by the National Center for Biotechnology Information: "The parent compound, coumarin, occurs naturally in many plants, natural spices, and foods such as tonka bean, cassia (bastard cinnamon or Chinese cinnamon), cinnamon, melilot (sweet clover), green tea, peppermint, celery, bilberry, lavender, honey (derived both from sweet clover and lavender), and carrots, as well as in beer, tobacco, wine, and other foodstuffs." It is also noted that coumarin, as well as its derivatives, has been used in a wide variety of dosing regimens for pharmaceutical uses, with no evidence of a cancer hazard.

Genotoxicity Studies: The weight of evidence demonstrates that coumarin is not a genotoxic agent. The results of the genotoxicity studies of coumarin are summarized in Tables 19 through 22 of the draft HID. These tables show that coumarin was not genotoxic in the majority of the genotoxicity and mutagenicity studies. Importantly, coumarin was negative in every single *in vivo* genotoxicity study, as shown in Table 22 of the draft HID. For example, coumarin was shown not to covalently bind DNA in target organs (liver and kidney) of both Sprague-Dawley and F344 rats. Comprehensive independent reviews of the data have concluded that coumarin is not genotoxic (EFSA 2004 and 2008, BfR 2003, NSCFS 2010).

CYP2A6 Polymorphisms and Toxicogenomics Data: Pages 46-63 and Appendix B of the draft HID provide interesting information on CYP2A6 polymorphisms in humans. This information provides little additional information of value for the purposes of identifying coumarin as a carcinogenic hazard since there are no epidemiological studies of coumarin and carcinogenicity. This information is more likely to be useful in risk assessment, not hazard identification, since it indicates differences in metabolism within the human population.

Similarly, pages 82-98 and Appendix C of the draft HID are devoted to toxicogenomic data on coumarin. The draft HID notes a toxicogenomics study found that coumarin up-regulated expression of seven cell-cycle related genes in rat liver. This information is not informative in determining whether coumarin poses a cancer hazard and as written is highly speculative and misleading. For example, the draft HID's summary of the

toxicogenomic data (Section 3.3.7.6), states that "OEHHA's functional pathways analysis show that multiple biological processes/pathways could be involved in the hepato-carcinogenicity of coumarin, such as glutathione metabolism, and the oxidative stress response." It is over-reaching to suggest that based on *in vitro* testing results at high concentrations that involvement of pathways for 'glutathione metabolism and the oxidative stress response' are in any way indicative of a cancer hazard. These are normal physiological processes that would be expected following exposures to many substances that are clearly not considered to be carcinogenic as part of the normal metabolic process.

Conclusion: The draft HID identifies a number of animal studies and no epidemiological studies that evaluated the potential carcinogenicity of coumarin. Further, the draft HID notes the quality and reliability of these studies varies. Many of these studies have significant limitations and shortcomings that render them not "scientifically valid testing according to generally accepted principles." The only clear evidence of carcinogenicity is the increased incidence of alveolar/bronchiolar adenomas and carcinomas among high dose female mice in the NTP bioassay. It is noted that the lung of the B6C3F1 mouse is known to be uniquely sensitive such that this tumor response has questionable relevance to humans. Clear evidence of a carcinogenic effect in one sex of one species in one study is not enough to list coumarin. The overall scientific evidence does not support a conclusion that coumarin has been clearly shown to cause cancer.

II. Introduction

These comments are submitted on behalf of the International Fragrance Association North America ("IFRA North America"). They set forth the scientific and regulatory reasons that coumarin does not meet the Proposition 65 criteria for listing: specifically, coumarin has not "been clearly shown through scientifically valid testing according to generally accepted principles to cause … cancer."[1] The Office of Environmental Health Hazard Assessment ("OEHHA") has provided a draft Hazard Identification Document ("draft HID") for coumarin. (OEHHA, 2017). This submission provides our comments to OEHHA and to the Proposition 65 Carcinogen Identification Committee (CIC) on the draft HID.

III. No Authoritative Body Has Formally Identified Coumarin as Causing Cancer.

No authoritative body has formally identified coumarin as causing cancer. OEHHA is required to place on the Proposition 65 list any substance that the U.S. EPA, U.S. FDA, IARC, NTP, or NIOSH – all authoritative bodies under Proposition 65 – have formally identified as causing cancer. Because coumarin cannot be listed on the basis of the views of any of these authoritative bodies, coumarin has been placed before the CIC to address the question of whether coumarin has "been clearly shown through scientifically valid testing according to generally accepted principles to cause … cancer." If the CIC were to identify coumarin as a carcinogen, we believe it would be the first scientific or regulatory body to do so.

No fewer than three Proposition 65 authoritative bodies (i.e., U.S. FDA, NTP and IARC) have been involved in the evaluation of the potential carcinogenicity of coumarin. The U.S. FDA and the NCI were responsible for nominating coumarin for carcinogenicity testing by the NTP. In response, the NTP conducted cancer bioassays of coumarin in rats

[1] California Health and Safety Code § 25249.8(b)

and mice, and the results of the NTP bioassays are not sufficient to list coumarin under the authoritative bodies listing mechanism of Proposition 65. NTP concluded:

> "Under the conditions of these 2-year gavage studies there was ***some evidence of carcinogenic activity**** of coumarin in male F344/N rats based on increased incidences of renal tubule adenomas. There was ***equivocal evidence of carcinogenic activity*** of coumarin in female F344/N rats based on a marginally - increased incidence of renal tubule adenomas. There was ***some evidence of carcinogenic activity*** of coumarin in male B6C3Fl mice based on the increased incidence of alveolar/bronchiolar adenomas. There was ***clear evidence of carcinogenic activity*** of coumarin in female B6C3Fl mice based on increased incidences of alveolar/bronchiolar adenomas, alveolar/bronchiolar carcinomas, and hepatocellular adenomas. The marginally increased incidences of squamous cell papillomas of the forestomach in male and female mice receiving 50 mg/kg may have been related to coumarin administration." (NTP, 1993, p.78.).

Under the authoritative bodies listing mechanism of Proposition 65, NTP must identify at least two instances of ***clear evidence of carcinogenic activity*** in NTP bioassays in rats and mice in order for a chemical to be listed. In the case of coumarin, NTP identified only one instance of ***clear evidence of carcinogenic activity***. Specifically, NTP found ***clear evidence of carcinogenic activity*** in female mice based on increased incidences of lung tumors (adenomas and carcinomas). But, since there was no ***clear evidence of carcinogenic activity*** in male mice or rats of either sex, the NTP's findings provide insufficient evidence to list coumarin as a carcinogen under the authoritative bodies listing mechanism.

And finally, in 2000, IARC evaluated the potential carcinogenicity of coumarin, and IARC classified coumarin as "not classifiable as to its carcinogenicity to humans (Group 3)." (IARC, 2000). In animals, IARC concluded: "There is *limited evidence* in experimental animals for the carcinogenicity of coumarin." In humans, IARC found, "No epidemiological data relevant to the carcinogenicity of coumarin were available."

Because IARC did not find sufficient evidence of carcinogenicity in humans or animals to warrant classification as at least a Group 2B chemical, coumarin cannot be listed via the authoritative bodies listing mechanism.

Importantly, all of the animal carcinogenicity studies of coumarin identified in the draft HID were available to the IARC Working Group when coumarin was evaluated in February 15-22, 2000. According to IARC (2000), "Coumarin has been adequately tested by oral administration in two experiments in mice [NTP, 1993; Carlton, 1996] and in one experiment in rats [NTP, 1993]." IARC stated that three other carcinogenicity studies in rats "could not be evaluated" due to various limitations. (IARC, 2000, p, 216). The limitations of these studies will be discussed later in these comments.[2] IARC also concluded that a carcinogenicity study of coumarin in hamsters (Ueno and Hirono, 1981) is "inadequate for evaluation." (IARC, 2000, p. 202).

Because no authoritative body has formally identified coumarin as causing cancer, OEHHA has asked the CIC to determine whether coumarin has "been clearly shown through scientifically valid testing according to generally accepted principles to cause … cancer."[3] Anything less does not permit listing under this statute. In reviewing the scientific data, the CIC must determine whether the stringent standard for Proposition 65 listing has been met for coumarin.

IV. Animal Studies Do Not Support a Finding that Coumarin Is Clearly Shown to Cause Cancer

The draft HID identifies carcinogenicity studies of coumarin in mice (2), rats (4), and hamsters (1). The quality and reliability of these studies varies. This section will include

[2] The three rat carcinogenicity studies of coumarin that were not considered adequate for evaluation by IARC were: (1) Carlton et al (1996), (2) Bar and Griepentrog (1967); Griepentrog (1973), (3) Hagan et al. (1967).
[3] California Health and Safety Code § 25249.8(b)

a description of the strengths and weaknesses of the key animal studies. Some of the studies identified in the draft HID do not constitute "scientifically valid testing according to generally accepted principles." The results of the animal studies do not support a conclusion that coumarin is clearly shown to cause cancer. Each of these studies is discussed separately in the sections below.

Carcinogenicity Studies in Mice

NTP (1993)

Evidence of the potential carcinogenicity of coumarin was observed in female B6C3F1 mice administered coumarin by gavage in the NTP bioassay. As noted in the draft HID, "Statistically significant increases in alveolar/bronchiolar adenomas, carcinomas, and adenomas and carcinomas combined were observed in the high-dose group compared to controls, with positive dose-response trends." (OEHHA, 2017, p, 23). The incidences of alveolar/bronchiolar carcinomas among female mice were 0/51, 0/49, 0/49, and 7/51 at 0, 50, 100, and 200 mg/kg, respectively. Of interest, these lung tumors were observed late in life (i.e., day of first tumor occurrence: 615), and they did not significantly reduce the survival of the high dose females. NTP concluded that there was "clear evidence of carcinogenic activity" in female mice based on the lung tumor data.

Importantly, B6C3F1 mice are particularly sensitive to lung tumors, which are the second most common spontaneous tumors observed in this strain of mice. The sensitivity of the mouse lung to coumarin is attributed to the high metabolic rate of coumarin epoxidation in the Clara cell. (Felter *et al.,* 2006) In comparison, the rat lung makes very little epoxide, and no Clara cell toxicity or increase in lung tumors is observed in this species. (Felter *et al,* 2006). As stated by Vassallo, *et al*, "Based on metabolic, anatomical, and morphological differences between rodents and humans, and the apparent metabolic requirements for toxicity in the mouse lung, the human lung is unlikely to be susceptible to coumarin-induced Clara cell toxicity." (Vassallo *et al.,* 2004b).

In male mice, NTP observed a statistically significant increase in alveolar/bronchiolar adenomas, but not carcinomas, at the high dose only. The incidences of alveolar/bronchiolar carcinomas among male mice were 1/50, 1/50, 2/50, and 1/51 at 0, 50, 100, and 200 mg/kg, respectively. This is important because the CIC has generally not identified substances as "clearly shown to cause cancer" that do not cause an increase in malignant tumors. As in female mice, these lung tumors appeared late in life (i.e., the day of first tumor occurrence: 716) without significantly affecting survival. NTP concluded there was "some evidence of carcinogenic activity" based on the increased incidence of benign lung tumors.

A statistically significant increase in hepatocellular adenomas (but not carcinomas) was seen in female mice in the NTP bioassay at the low and middle dose levels, but not at the high dose. In males, no statistically significant increase in hepatocellular adenomas or carcinomas was observed at any dose.

And finally, forestomach tumors (primarily benign) were marginally increased among male and female mice at the low dose, but not at the middle and high doses. NTP also noted:

> "Further, the incidences in the 50 mg/kg groups slightly exceeded the highest incidences observed in groups of historical controls. However, the forestomach neoplasms could not be clearly attributed to the administration of coumarin because the incidences in the 50 mg/kg groups were not significantly greater than those of the controls and there was no corresponding increase over a fourfold range of doses from 50 to 200 mg/kg." (NTP, 1993, p.77)

Carlton et al. (1996)

In a carcinogenicity study in CD-1 mice, Carlton et al. (1996) administered coumarin in the diet at doses of 0, 300, 1000, and 3000 ppm. According to the study authors, these concentrations provided doses of 28, 91, and 271 mg/kg/day and 26, 86, and 280

mg/kg/day for female and male mice, respectively. Body weight gain of male mice receiving the high dose was significantly reduced compared to controls, exhibiting an 18% reduction over the first 52 weeks of the study. A similar decrease in body weight gain was not observed among high dose females.

In contrast to the NTP bioassay, no dose-related increase in pulmonary adenomas or carcinomas (assumed to be equivalent to alveolar/bronchiolar tumors described by NTP) was observed in female mice, despite the fact that Carlton included a higher dose level of coumarin than did NTP. Thus, the increased incidence of lung tumors observed among high dose female mice in the NTP bioassay was not confirmed in the Carlton et al. study.

In female mice, the draft HID reported a statistically significant increase in combined hepatocellular adenomas and carcinomas at the low dose, but not at the middle and high dose levels in the Carlton et al. study. The study authors stated that "all tumor incidences were within laboratory historic control values for this age and strain of mice." (Carlton, 1996). Thus, female mice in the Carlton et al. study did not clearly show that coumarin causes cancer.

In male mice, no significant increase in liver tumors was observed at any dose level. The incidences of hepatocellular adenomas and carcinomas combined were 20/52, 22/52, 19/52, and 12/52 at 26, 86, and 280 mg/kg/day. The draft HID reported that the incidence of pulmonary carcinoma, but not pulmonary adenoma, was statistically significantly increased among male mice administered the high dose of coumarin based on a Fisher pairwise comparison with controls conducted by OEHHA. (OEHHA, 2017, p. 27). Although no information was provided on statistical evaluation of the lung tumors in the Carlton et al. publication, the IARC Working Group "was aware of an unpublished company report in which statistical analyses had been applied to mortality-adjusted tumour rates. The Fisher's exact test for differences between treatment groups and Mantel's test for dose-related trends showed no treatment-related effect for any tumour type." (IARC, 2000, p. 198). Without the individual animal data survival and tumors, OEHHA would not have been able to evaluate the mortality-adjusted tumor rates.

Importantly, the study authors stated that "all tumor incidences were within laboratory historic control values for this age and strain of mice." (Carlton, *et al.,* 2006).

In summary, the incidence of tumors, including lung and liver tumors, did not exceed the historical control range at any dose in either male or female mice. No dose-related effect on liver tumors was observed in mice of either sex. Although a statistically significant increase in pulmonary carcinoma was described in the draft HID among high dose males based on a statistical analysis conducted by OEHHA, there was no statistically significant effect when the tumor data was adjusted for mortality, as described by IARC. Thus, there was no clear evidence of a treatment-related effect on lung, liver, or any other type of tumors in male or female CD-1 mice administered coumarin in the diet.

Carcinogenicity Studies in Rats

NTP (1993)

Compared to controls, male F344/N rats administered 50, 100, and 200 mg/kg by gavage exhibited a small increase in the incidence of renal tubule adenomas (but not carcinomas), which was seen only after step-sectioning of the kidneys. But, there was no apparent dose-response relationship. The incidence of renal tubule adenomas (single sections and step-sections combined) observed among male rats was 1/49, 6/50, 7/51, and 5/50 at 0, 50, 100 and 200 mg/kg, respectively. (NTP, 1993, p. 43). The increases were statistically significant by pairwise comparison at the low and middle dose levels, but not at the high dose. In addition, these benign tumors were observed in the presence of a high incidence of severe nephropathy, which is common in male F344/N rats. In fact, the incidence of nephropathy ranged from 96% to 100% among the treatment groups, and the severity of nephropathy was increased in a dose-related manner. Only one renal tubule carcinoma was observed among male rats, and it was seen in a low dose male (0/49, 1/50, 0/51, and 0/50 at 0, 50, 100 and 200 mg/kg, respectively). NTP concluded that there was "some evidence of carcinogenic activity of coumarin in male F344/N rats based on increased incidence of renal tubule adenomas." (NTP, 1993, p. 78).

In female rats, NTP reported no significant effect (by pairwise comparison) on renal tubule adenomas or carcinomas at any dose. No renal tubule carcinomas were observed at any dose among the female rats in the NTP bioassay. The incidence of renal tubule adenomas among female rats was 0/49, 0/50, 1/50, and 2/49 at 0, 50, 100, and 200 mg/kg, respectively. In addition, NTP reported that the trend test was not statistically significant ($p > 0.05$) for these benign tumors. However, OEHHA reported a trend test p value of 0.0489 for the incidence of renal tubule adenomas when OEHHA used the "number of tumor-bearing animals per number of animals alive at time of first occurrence of tumor (day 699)" in the denominator. OEHHA also reported that renal tubule adenomas are rare in female rats based on NTP's historical control data; however, it should be noted that the results in the coumarin bioassay were based on the results of single and step-sectioning (combined) of the kidneys whereas most historical controls data came from studies which did not employ step-sectioning of the kidneys. The incidence of nephropathy among female rats was lower and of lesser severity than that observed in male rats. NTP concluded that there was "equivocal evidence of carcinogenic activity of coumarin in female F344/N rats based on a marginally increased incidence of renal tubule adenomas." (NTP, 1993, p. 78). In female rats, there was no statistically significant (by pairwise comparison) effect on renal tubule adenomas incidence of renal tubule adenomas (0/49, 0/50, 1/50, and 2/49 at 0, 50, 100, and 200 mg/kg, respectively).

Carlton et al. (1996)

In the Carlton et al. (1996) rat carcinogenicity study, no significant increase in the incidence of any type of tumor was observed among male and female Sprague-Dawley rats exposed to dose levels of coumarin that did not exceed the Maximum Tolerated Dose (MTD). In comparison, increases in liver tumors were reported at doses which clearly exceeded the MTD. However, dose levels that exceed the MTD are not considered to represent "scientifically valid testing according to generally accepted principles." Carlton et al. (1996) administered to rats' diets containing 0, 333, 1000, 2000, 3000, or 5000 ppm of coumarin for two years (achieving dose levels of 0, 13, 42, 87, 130 or 235

mg/kg/day in males and 0, 16, 50, 107, 156, or 283 mg/kg/day in females). In addition, rats receiving all but the two highest dose levels were also exposed *in utero* and throughout lactation.

Increased incidences of liver tumors (i.e., cholangiocarcinoma and parenchymal tumors) were observed among male and female rats receiving 5000 ppm of coumarin, a dose which greatly exceeds the Maximum Tolerated Dose (MTD). At 5000 ppm of coumarin, the mean body weight in the high-dose group was approximately 43% lower than controls at 52 weeks and 35% lower at 104 weeks. Food consumption was also drastically reduced at 5000 ppm in both male and female rats.

At 3000 ppm, a single cholangiocarcinoma in a male rat was considered by the study authors to be potentially treatment-related, although it is impossible to determine this based on a single tumor, and it is noted that there was no increase in cholangiocarcinomas in the NTP bioassays. Body weight gain and food consumption were statistically significantly reduced at both 2000 and 3000 ppm, but to a lesser extent than was observed at 5000 ppm. Administration of coumarin in the diet did not produce an increase in any type of tumor at concentrations of 2000 ppm or less.

The study authors concluded:

> "Given body weight decrements up to 60% among high-dose male rats, clearly indicating that the maximum tolerated dose was exceeded, the carcinogenic response seen in the current Sprague-Dawley rat study is likely due to the hepatotoxicity incurred following high-dose exposure. Tumors were not metastatic and survival was significantly increased among rats in the two highest dose groups." (Carlton *et al.,* 1996, p. 150).

The draft HID theorized that the highest dose groups in the Carlton et al. (1995) may not have exceeded the MTD:

> "Body weight gain was decreased in the three highest dose groups in this study, this, however, is not by itself an indication of an excessive high dose. It is possible that a reduction in food consumption and consequent reduced body weight gain may have contributed to the greater survival rates observed in the highest two dose groups. Feed restriction studies have shown that reduced body weight is associated with increased survival and reductions in spontaneous liver tumor incidence (NTP, 1997). Yet in this study, increased incidences of treatment-related liver tumors were observed." (OEHHA, 2017, p, 16).

However, whether the decreased body weights were due to excessive toxicity or simply due to reduced food consumption due to reduced palatability begs the obvious issue. Rats with body weights approximately half of those of the controls at the end of the first year are not acceptable for scientifically valid testing. Yes, there are data to indicate that reduced food consumption lowers the incidence of certain types of tumors. Also, the increased survival of the rats at the highest dose levels also provides a longer period of time for tumors to develop. Dr. Joseph Haseman and others have developed models to predict the impact of reduced body weight on the incidence of certain tumors. But, no models have been developed for rats with a 60% decrease in body weight compared to controls after one year because body weight reductions of such a magnitude are not normally seen in carcinogenicity bioassays in animals. It is preposterous to argue that a dose level that causes a 60% reduction in body weight does not exceed the MTD for a cancer bioassay. Regulatory guidelines generally consider reductions in body weight greater than 10-15% to exceed the MTD.[4] In short, the highest dose levels in the Carlton et al. (1996) rat carcinogenicity should not be considered "scientifically valid testing according to generally accepted principles."

It is also interesting to note that IARC's Working Group concluded that the Carlton et al. (1996) rat study was inadequate for IARC's evaluation:

[4] For example, see OECD (2002) Guidance Notes for Analysis and Evaluation of Chronic Toxicity and Carcinogenicity Studies. OECD Series on Testing and Assessment No. 35, OECD, Paris. Available at: http://www.olis.oecd.org/olis/2002doc.nsf/LinkTo/NT00002BE2/$FILE/JT00130828.PDF.

> "Coumarin has been adequately tested by oral administration in two experiments in mice and **in one experiment in rats**. … Three other studies in rats could not be evaluated." [emphasis added] (IARC, 2000, p. 216).

The one study in rats described by the IARC Working Group as "adequately tested" is the NTP (1993) cancer bioassay in rats. The Carlton et al. (1996) study was one of the "three other studies in rats that could not be evaluated," according to the IARC. The IARC Working Group noted: "Dose-related decreases in body weight gain in excess of 10–15% occurred in the 2000-, 3000- and 5000-ppm dose groups." The IARC Working Group also noted "the unusually sharp increase in tumour incidence in the liver at only the highest of five doses and the lack of adequate histopathological description of both tumour types. Given the issue related to misdiagnosis of bile duct tumours in an earlier study [by Bar and Griepentrog, 1967], the Working Group was concerned that no descriptive information or illustrations were provided to confirm the diagnosis of cholangiocarcinoma, nor a discussion of the pathology." (IARC, 2000, p. 216).

Given these findings, the choice of supra-MTD dose levels and pathological conclusions in the Carlton et al. (1996) should not be considered "scientifically valid testing according to generally accepted principles."

Hagan et al. (1967)

The Hagan et al. (1967) study is inadequate for carcinogenicity evaluation. IARC excluded this study from its evaluation. The draft HID states: "This study was limited by the small number of animals per group and inadequate reporting."

Hagan et al. (1967) administered coumarin in the diet at 0, 1000, 2500, or 5000 ppm to groups of 5 to 7 male and female Osborne-Mendel rats for two years. No treatment-related tumors were reported. But the study suffers from many limitations and deficiencies. It should not be considered "scientifically valid testing according to generally accepted principles."

Bar and Griepentrog (1967); Griepentrog (1973)

The Bär and Griepentrog (1967) study (also reported in Griepentrog, 1973) is inadequate for carcinogenicity evaluation. The IARC Working Group excluded this study from its evaluation. (IARC, 2000). The draft HID identified multiple limitations: "The study reported the tumor incidences for male and female rats combined. The original study reported that bile duct carcinomas were observed in 12 rats in the 5000 ppm group and 5 rats in the 6000 ppm group. Reporting of histopathology was ambiguous and inconsistent between the two papers, information on other toxic endpoints and body weights is lacking, and there was no information on the purity of the coumarin administered." (OEHHA, 2017, p. 20).

Coumarin was administered to male and female albino rats (strain not specified) in feed containing 0, 1000, 2500, 5000, or 6000 ppm coumarin for up to two years (20 to 32 rats per group) (Bär and Griepentrog, 1967; Griepentrog, 1973). The study authors reported that exposure to coumarin to concentrations of 5000 ppm or greater induced bile duct carcinoma. However, as noted in the draft HID: "In a re-evaluation of the slides by external pathologists, the bile duct carcinomas were reclassified as non-neoplastic cholangiofibrosis (Cohen, 1979)." A decade later, another reevaluation of the original histopathology slides by Evans *et al.* (1989) confirmed that cholangiofibrosis and not cholangiocarcinoma was present.

In summary, the Bar and Griepentrog (1967) study, including the Griepentrog (1973) publication, should not be regarded as "scientifically valid testing according to generally accepted principles."

Carcinogenicity Studies in Hamsters

Ueno and Hirono (1981)

The study in hamsters by Ueno and Hirona (1981) is inadequate for carcinogenicity hazard identification. The IARC Working Group excluded this study from its evaluation in 2000. (IARC, 2000). The draft HID states the utility of this study for assessing the carcinogenicity of coumarin is limited by the small numbers of animals per group and poor survival in the low dose group (males) and in the control and treated groups (females)." (OEHHA, 2017, p. 29).

Ueno and Hirono (1981) administered coumarin to 8-week old male and female Syrian golden hamsters via diet at levels of 0, 1000 and 5000 ppm for up to 2 years. The group size was inadequate, ranging from 11-12 among males and 10-13 among females. No statistically significant increase in any type of tumors was observed in the treated groups; however, two pancreatic islet cell carcinomas were observed in the high dose group, but not in the control or the low dose groups.

In short, the Ueno and Hirono (1981) study should not be regarded as "scientifically valid testing according to generally accepted principles."

V. Human Studies Do Not Support a Finding that Coumarin Is Clearly Shown to Cause Cancer

No epidemiological studies on the potential carcinogenicity of human exposure to coumarin were identified in recent literature searches conducted by OEHHA and IFRA North America. (OEHHA, 2017, p. 7). It is noted that coumarin is naturally occurring in a number of plants, including many that are a common part of the human diet (e.g., strawberries, apricots, cherries). As summarized by the National Center for Biotechnology Information: "The parent compound, coumarin, occurs naturally in many plants, natural spices, and foods such as tonka bean, cassia (bastard cinnamon or Chinese cinnamon), cinnamon, melilot (sweet clover), green tea, peppermint, celery, bilberry, lavender, honey (derived both from sweet clover and lavender), and carrots, as well as in

beer, tobacco, wine, and other foodstuffs."[5] It is also noted that coumarin, as well as its derivatives, has been used in a wide variety of dosing regimens for pharmaceutical uses, with no evidence of a cancer hazard.

VI. Genotoxicity Studies Do Not Support a Finding that Coumarin Is Clearly Shown to Cause Cancer

The weight of evidence clearly demonstrates that coumarin is not a genotoxic agent. Comprehensive independent reviews of the data have also concluded that coumarin is not a genotoxic chemical (EFSA 2004 and 2008, BfR 2003, NSCFS 2010).

The results of the genotoxicity studies of coumarin are summarized in the draft HID in Tables 19 through 22. Importantly, coumarin was negative in every single *in vivo* genotoxicity study, as shown in Table 22 of the draft HID. For example, coumarin was shown not to covalently bind DNA in target organs (liver and kidney) of both Sprague-Dawley and F344 rats (Swenberg, 2003) and a lack of genotoxicity has also been demonstrated in studies of unscheduled DNA synthesis (UDS, OECD 482; Edwards, 2000). Furthermore, three *in vivo* micronucleus assays (OECD 474) were also negative in Swiss, IRC and B6C3F_1 mouse strains following exposure to coumarin (Api, 2001; Morris and Ward, 1992; NTP, 1993).

While some *in vitro* studies of coumarin demonstrate genotoxic effects, such results are not congruent with the results of *in vivo* studies. In Ames assays, no evidence of mutagenicity was demonstrated when coumarin was evaluated in *S. typhimurium* strains TA98, TA1535, TA1537 and TA1538, both with or without metabolic activation. However, with metabolic activation, high concentrations of coumarin demonstrated a weak positive effect in strain TA100, but only under non-standard conditions (Haworth, 1983). The relevance of this weak positive effect in strain TA100 only is questionable, especially since a biologically relevant (generally accepted threshold for TA 100: 2-fold increase vs control) response was only observed from samples co-exposed with the liver

[5] https://pubchem.ncbi.nlm.nih.gov/compound/323#section=Top

S9 fraction from Aroclor 1254 treated Syrian hamsters (Haworth et al., 1983). Arochlor induced Hamster S9 is not being used anymore, and uninduced hamster S9 is only indicated for chemicals that require reductive conditions, like, e.g., cleavage of an aromatic azo-bond (OECD 471, 1997; Prival and Mitchell, 1981). The positive result in the presence of Hamster S9 is also puzzling since the Syrian hamster appears to be resistant to coumarin-induced hepatotoxicity (Lake, 1992). The questionable finding in the Ames must be balanced with the favorable outcome in the aforementioned in vivo studies. If coumarin was a relevant mutagen in vivo it would be expected to show activity in potential target organs of coumarin. It did not - it tested clearly negative in the liver UDS test in rats (Edwards, 2000) and was demonstrated not to covalently bind DNA in liver and kidney of Sprague-Dawley and F344 rats (Swenberg, 2003).

Studies which investigated the ability of coumarin to induce clastogenic activity in vitro gave mixed results. This is not an unusual finding since in vitro clastogenicity assays have been associated with a high percentage of irrelevant positive findings (Kirkland *et al.,* 2005, Matthews *et al.,* 2006). The respective positive results, which mostly stem from non-standard, non-guideline compliant experiments, are balanced with favorable results from three micronucleus studies in mice which were performed with doses that are consistent with, or exceed, the maximal dose used in the mouse bioassays. Favorable results from these higher tier assays therefore demonstrate that the observed (questionable) in vitro clastogenic activity does not translate to the in vivo situation.

Taken together, the weight of evidence of all available data clearly points towards the absence of an in vivo genotoxic potential of coumarin.

Note: Table 21 of the draft HID summarizes the results of *in vitro* genotoxicity studies by Costa Rde et al. (2008) of extracts/infusions of the South American medicinal plant Mikania glomerata, known as “guaco”. While these test materials may contain coumarin as a component, they would also contain many other chemicals extracted from this medicinal plant. As a result, it is not possible to draw any conclusions about the *in vitro* genotoxicity of coumarin from these studies.

VII. Coumarin Is Unlikely to Pose a Carcinogenic Hazard to Humans Due to Differences in Metabolism

There are significant quantitative differences in the metabolism of coumarin between humans and rodents. In all species, coumarin undergoes extensive metabolism, as most coumarin metabolites are excreted and no significant accumulation of coumarin in tissues has been demonstrated (Lake, 1999). As shown in Figure 1, coumarin may be metabolized by hydroxylation at all six possible positions and by opening of the lactone ring and cleavage of carbon atom 2, generating carbon dioxide and various products including *o*-hydroxyphenylacetaldehyde (*o*-HPA), o-hydroxyphenylethanol (*o*-HPE), and *o*-hydroxyphenylacetic acid (*o*-HPAA). The two primary pathways of coumarin metabolism are 7-hydroxylation and the 3,4-coumarin epoxidation pathway. Comparative toxicokinetic and metabolism and molecular modeling studies have demonstrated marked species differences in the metabolism of coumarin (Cohen, 1979; Fentem and Fry, 1993; Lake, 1999, Born, 2000, Lewis and Lake, 2002). Additionally, *in vitro* analysis of 7-hydroxylase activity in liver microsomes, suggest that 7-hydroxylation is a minor pathway of coumarin metabolism in many species (e.g. the rat, mouse, Syrian hamster, gerbil and guinea pig; Lake et al., 1992a; Fentem and Fry, 1992).

In rats, mice, and other rodents the predominant biotransformation pathway of coumarin is the 3,4-coumarin epoxidation pathway. *In vitro* studies have demonstrated that *o*-HPA, the major metabolite of coumarin in the rat and mouse, is more toxic to rat hepatocytes than the parent compound and other coumarin metabolites (Born et al., 1998; Lake et al., 1999). Once the unstable coumarin intermediate 3,4-epoxide is formed, it is rapidly converted to the hepatotoxic metabolite *o*-HPA (the major coumarin metabolite found in liver microsomes of rats and mice). *o*-HPA may subsequently be converted to *o*-HPAA and *o*-HPE (Norman & Wood, 1984; Fentem et al., 1991; Lake et al., 1992a,b). The slow detoxification process of *o*-HPA in rats compared to other species explains why, in long-term studies, liver toxicity and hepatic tumors were only observed in rats (Lake, 1999).

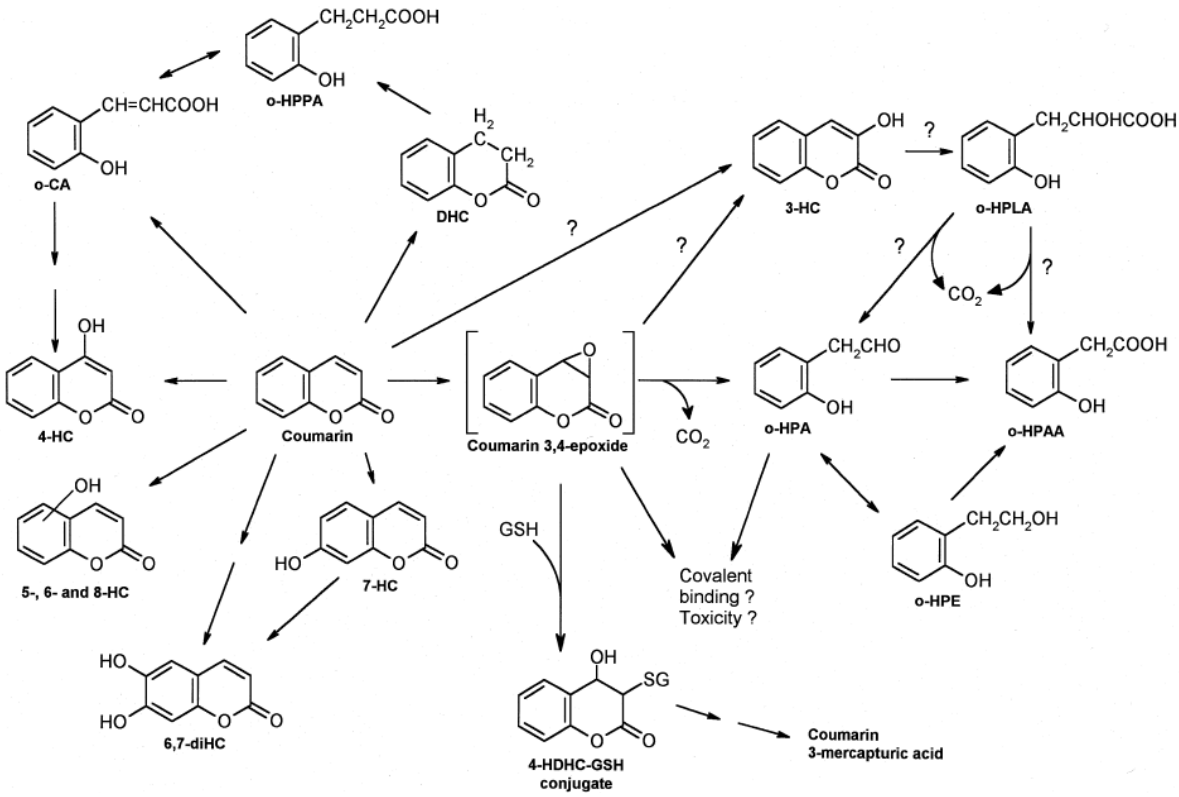

Figure 1: Some Pathways of Coumarin Metabolism (Lake, 1999)

With regards to the species-specific increase in lung toxicity and carcinogenicity in mice; 3,4-coumarin epoxide formation occurs in mouse lung Clara cells which are found in relatively higher abundance at the terminal bronchiolar regions in the lung (NTP, 1993; Born *et al.,* 1998; Vassallo *et al.,* 2004b).

The predominant metabolic pathway of coumarin in humans is 7-hydroxycoumarin (7-HC; a detoxification pathway), mediated by the CYP2A6 enzyme. This pathway does not generate toxic metabolites. The primary metabolite 7-HC is rapidly excreted in urine (half-life of 1-2 hours in humans) as glucuronic acid and sulfate conjugates. While most studies with human liver microsomes focus on 7-hydroxylation, the formation of other coumarin metabolites, including products of the 3,4-epoxidation have also been observed suggesting that human liver microsomes are capable of metabolizing coumarin through alternative pathways (Fentem and Fry, 1992; Lake et al., 1992a). In evaluating these *in*

vitro studies, it is important to consider the relevance of the substrate concentration used compared to the *in vivo* situation because several studies have demonstrated low Km values for coumarin 7-hydroxylase activity in human liver microsomes (Fentem and Fry, 1992; Koenigs et al., 1997; Pearce et al., 1992; Shimada et al., 1996). This high affinity of CYP2A6 for coumarin implies that at low, physiologically relevant, substrate concentrations 7-HC formation will be the major pathway of coumarin metabolism in the livers of most humans whereas, at high coumarin substrate concentrations the 7-hydroxylation pathway is saturated and greater amounts of other metabolites are observed. (Lake, 1999).

In summary, coumarin is much less likely to pose a carcinogenic hazard to humans compared to rodents due to the significant quantitative differences in metabolic pathways. Further, it has not been clearly demonstrated that coumarin causes cancer even to rodents.

VIII. Data on Polymorphisms and Toxicogenomics Do Not Support a Finding that Coumarin Is Clearly Shown to Cause Cancer

Pages 46-63 and Appendix B of the draft HID provide interesting information on CYP2A6 polymorphisms in humans. This information provides little additional information of value for the purposes of identifying coumarin as a carcinogenic hazard since there are no epidemiological studies of coumarin and carcinogenicity. This information is more likely to be useful in risk assessment, not hazard identification, since it indicates differences in metabolism within the human population.

Similarly, pages 82-98 and Appendix C of the draft HID are devoted to toxicogenomic data on coumarin. The draft HID notes a toxicogenomics study found that coumarin up-regulated expression of seven cell-cycle related genes in rat liver. This information is not informative in determining whether coumarin poses a cancer hazard and as written is highly speculative and misleading. For example, the draft HID's summary of the toxicogenomic data (Section 3.3.7.6), states that "OEHHA's functional pathways analysis show that multiple biological processes/pathways could be involved in the hepato-

carcinogenicity of coumarin, such as glutathione metabolism, and the oxidative stress response. In addition, as shown in Table 28, there are several common cancer-related biological processes/pathways altered by coumarin in rat liver and in human primary hepatocytes, including up-regulated pathways related to nucleic acid binding, and protein binding, and down-regulated pathways related to metabolism of xenobiotics by CYPs, oxidoreductase activity, and mitochondrial functions." It is over-reaching to suggest that based on *in vitro* testing results at high concentrations that involvement of pathways for 'glutathione metabolism and the oxidative stress response' are in any way indicative of a cancer hazard. These are normal physiological processes that would be expected following exposures to many constituents as part of the normal metabolic process.

IX. CONCLUSION

The draft HID identifies a number of animal studies and no epidemiological studies that evaluated the potential carcinogenicity of coumarin. As the draft HID notes, the quality and reliability of these studies varies. Many of these studies have significant limitations and shortcomings that render them not "scientifically valid testing according to generally accepted principles." The only clear evidence of carcinogenicity is the increased incidence of alveolar/bronchiolar adenomas and carcinomas among high dose female mice in the NTP bioassay. Clear evidence of a carcinogenic effect in one sex of one species in one study is not enough to list coumarin. The overall scientific evidence does not support a conclusion that coumarin has been clearly shown to cause cancer.

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